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ENTITLED

METHOD FOR EXTENDING THE DYNAMIC DETECTION RANGE OF ASSAY DEVICES

BY

XUEDONG SONG

METHOD FOR EXTENDING THE DYNAMIC DETECTION RANGE OF ASSAY DEVICES

Background of the Invention

Various analytical procedures and devices are commonly employed in flow-through assays to determine the presence and/or concentration of analytes that may be present in a test sample. For instance, immunoassays utilize mechanisms of the immune systems, wherein antibodies are produced in response to the presence of antigens that are pathogenic or foreign to the organisms. These antibodies and antigens, i.e., immunoreactants, are capable of binding with one another, thereby causing a highly specific reaction mechanism that may be used to determine the presence or concentration of that particular antigen in a biological sample.

There are several well-known immunoassay methods that use immunoreactants labeled with a detectable component so that the analyte may be detected analytically. For example, "sandwich-type" assays typically involve mixing the test sample with detectable probes, such as dyed latex or a radioisotope, which are conjugated with a specific binding member for the analyte. The conjugated probes form complexes with the analyte. These complexes then reach a zone of immobilized antibodies where binding occurs between the antibodies and the analyte to form ternary "sandwich complexes." The sandwich complexes are localized at the zone for detection of the analyte. This technique may be used to obtain quantitative or semi-quantitative results. Some examples of such sandwich-type assays are described in. by U.S. Patent Nos. 4,168,146 to Grubb, et al. and 4,366,241 to Tom, et al. An alternative technique is the "competitive-type" assay. In a "competitive-type" assay, the label is typically a labeled analyte or analyte-analogue that competes for binding of an antibody with any unlabeled analyte present in the sample. Competitive assays are typically used for detection of analytes such as haptens, each hapten being monovalent and capable of binding only one antibody molecule. Examples of competitive immunoassay devices are described in U.S. Patent Nos. 4,235,601 to Deutsch, et al., 4,442,204 to Liotta, and 5,208,535 to Buechler, et al.

Despite the benefits achieved from these devices, many conventional lateral flow assays encounter significant inaccuracies when exposed to relatively high

analyte concentrations. For example, assays that rely on optical detection (e.g., fluorescence, reflectance, phosphorescence, etc.) often become inaccurate at high analyte concentrations. Specifically, the probes are usually not only captured on the surface of the membrane device, but also within the interior of the assay device. Unfortunately, most optical detection techniques are not capable of detecting those probes captured deep within the interior of the assay device. In addition, fluorescent probes sometimes exhibit "self-quenching" when placed too close together. Self-quenching is a well-known phenomenon that occurs when two or more fluorescent materials interact photochemically to quench each other's fluorescence. Thus, fluorescent probes may begin to exhibit self-quenching at high analyte concentrations, which actually results in a decrease in the fluorescent intensity. Those problems often limit the detection range and result in an inaccurate detection of an analyte.

In response to these or other problems, several assays configurations have been proposed. For example, EP 0462376 to Ching describes an assay device that includes a solid phase having at least two defined and marked detection sites in sequential fluid-flow contact. The first detection site is a capture site immobilized with a capture reagent capable of competing with the analyte for binding to a conjugate. A second detection site is a conjugate recovery site that includes a conjugate recovery agent different from the capture reagent for binding to the conjugate or a complex thereof that passes through the capture site. As the amount of analyte in the test sample increases, the more the bonding sites of the conjugate are occupied by analyte molecules and the less the conjugate is free to bind to the capture reagent. Instead, the analyte/conjugate complexes pass through the capture site and migrate into the conjugate recovery site. A comparative analysis of the amounts of label at each site indicates the amount of analyte in the test sample.

Nevertheless, a need still exists for a method of extending the dynamic detection range of an assay device in an accurate, yet simple and cost-effective manner.

Summary of the Invention

In accordance with one embodiment of the present invention, a flow-through assay device for detecting the presence or quantity of an analyte residing in a test

sample is disclosed. The flow-through assay device comprises a porous membrane that is in communication with detection probes and calibration probes, the detection probes being conjugated with a specific binding member for the analyte. If desired, the conjugated detection probes may comprise a substance selected from the group consisting of chromogens, catalysts, luminescent compounds (e.g., fluorescent, phosphorescent, etc.), radioactive compounds, visual labels, liposomes, and combinations thereof. The specific binding member may be selected from the group consisting of antigens, haptens, aptamers, primary or secondary antibodies, biotin, and combinations thereof.

The porous membrane defines a detection zone within which a first capture reagent is immobilized that is configured to bind to at least a portion of the conjugated detection probes or complexes thereof to generate a detection signal having an intensity. In one embodiment, the first capture reagent is selected from the group consisting of antigens, haptens, protein A or G, neutravidin, avidin, streptavidin, captavidin, primary or secondary antibodies, and complexes thereof. For instance, the first capture reagent may bind to complexes formed between the analyte and the conjugated detection probes.

To further extend the dynamic detection range of the assay device, the porous membrane also defines a compensation zone located downstream from the detection zone. A second capture reagent is immobilized within the compensation zone that is configured to bind to the conjugated detection probes or complexes thereof passing through the detection zone to generate a compensation signal having an intensity. In one embodiment, the second capture reagent is selected from the group consisting of antigens, haptens, protein A or G, neutravidin, avidin, streptavidin, captavidin, primary or secondary antibodies, and complexes thereof. In another embodiment, the second capture reagent comprises a polyelectrolyte. The polyelectrolyte may be positively charged, negatively charged, amphiphilic, etc. Regardless of the material selected for the second capture reagent, the intensity of the compensation signal is inversely proportional to the intensity of the detection signal. Accordingly, the ratio of the detection signal intensity to the compensation signal intensity is proportional to analyte concentration, and thus may be used for determining the amount of the analyte in the test sample.

The accuracy of the detection and compensation signals under actual test

conditions may be further improved using self-calibration techniques. Specifically, the porous membrane is also in communication with calibration probes and comprises a calibration zone within which a third capture reagent is immobilized that is configured to bind to the calibration probes to generate a calibration signal having an intensity. The calibration signal is substantially constant in intensity relative to the intensities of the detection and compensation signals. Thus, the calibration signal may be used to calibrate the detection and compensation signals.

In accordance with another embodiment of the present invention, a method for detecting the presence or quantity of an analyte residing in a test sample is disclosed. The method comprises:

i) providing a flow-through assay device comprising a porous membrane, the porous membrane being in communication with detection probes and calibration probes, the detection probes being conjugated with a specific binding member for the analyte, the porous membrane defining a detection zone within which a first capture reagent is immobilized, a compensation zone within which a second capture reagent is immobilized, and a calibration zone within which a third capture reagent is immobilized, wherein the compensation zone is located downstream from the detection zone;

ii) contacting a test sample containing the analyte with the conjugated detection probes and the calibration probes;

iii) measuring a detection signal intensity generated at the detection zone, a compensation signal intensity generated at the compensation zone, and a calibration signal intensity generated at the calibration zone;

iv) comparing the intensity of the detection signal to the compensation signal, wherein the intensity of the compensation signal is inversely proportional to the intensity of the detection signal; and

v) calibrating the compared intensities of the detection signal and the compensation signal with the intensity of the calibration signal, wherein the intensity of the calibration signal is substantially constant relative to the intensities of the detection signal and the calibration signal. If desired, the method may further comprise generating a calibration curve by plotting the ratio of the detection signal intensity to the compensation signal intensity calibrated by the intensity of

the calibration signal for a plurality of predetermined analyte concentrations.

Other features and aspects of the present invention are discussed in greater detail below.

Brief Description of the Drawings

A full and enabling disclosure of the present invention, including the best mode thereof, directed to one of ordinary skill in the art, is set forth more particularly in the remainder of the specification, which makes reference to the appended figures in which:

Fig. 1 is a perspective view of one embodiment of a flow-through assay device of the present invention;

Fig. 2 is a graphical illustration of one embodiment for covalently conjugating an antibody to a detection probe;

Fig. 3 is graphical illustration of the relationship between analyte concentration and signal intensities for the detection and compensation zones in accordance with one embodiment of the present invention; and

Fig. 4 is a graphical illustration of the mechanism used for one embodiment of a sandwich assay format of the present invention.

Repeat use of reference characters in the present specification and drawings is intended to represent same or analogous features or elements of the invention.

Detailed Description of Representative Embodiments

Definitions

As used herein, the term "analyte" generally refers to a substance to be detected. For instance, analytes may include antigenic substances, haptens, antibodies, and combinations thereof. Analytes include, but are not limited to, toxins, organic compounds, proteins, peptides, microorganisms, amino acids, nucleic acids, hormones, steroids, vitamins, drugs (including those administered for therapeutic purposes as well as those administered for illicit purposes), drug intermediaries or byproducts, bacteria, virus particles and metabolites of or antibodies to any of the above substances. Specific examples of some analytes include ferritin; creatinine kinase MB (CK-MB); digoxin; phenytoin; phenobarbitol; carbamazepine; vancomycin; gentamycin; theophylline; valproic acid; quinidine; luteinizing hormone (LH); follicle stimulating hormone (FSH); estradiol,

progesterone; C-reactive protein; lipocalins; IgE antibodies; cytokines; vitamin B2 micro-globulin; glycated hemoglobin (Gly. Hb); cortisol; digitoxin; N-acetylprocainamide (NAPA); procainamide; antibodies to rubella, such as rubella-IgG and rubella IgM; antibodies to toxoplasmosis, such as toxoplasmosis IgG (Toxo-IgG) and toxoplasmosis IgM (Toxo-IgM); testosterone; salicylates; acetaminophen; hepatitis B virus surface antigen (HBsAg); antibodies to hepatitis B core antigen, such as anti-hepatitis B core antigen IgG and IgM (Anti-HBC); human immune deficiency virus 1 and 2 (HIV 1 and 2); human T-cell leukemia virus 1 and 2 (HTLV); hepatitis B e antigen (HBeAg); antibodies to hepatitis B e antigen (Anti-HBe); influenza virus; thyroid stimulating hormone (TSH); thyroxine (T4); total triiodothyronine (Total T3); free triiodothyronine (Free T3); carcinoembryoic antigen (CEA); lipoproteins, cholesterol, and triglycerides; and alpha fetoprotein (AFP). Drugs of abuse and controlled substances include, but are not intended to be limited to, amphetamine; methamphetamine; barbiturates, such as amobarbital, secobarbital, pentobarbital, phenobarbital, and barbital; benzodiazepines, such as librium and valium; cannabinoids, such as hashish and marijuana; cocaine; fentanyl; LSD; methaqualone; opiates, such as heroin, morphine, codeine, hydromorphone, hydrocodone, methadone, oxycodone, oxymorphone and opium; phencyclidine; and propoxyhene. Other potential analytes may be described in U.S. Patent Nos. 6,436,651 to Everhart, et al. and 4,366,241 to Tom et al.

As used herein, the term "test sample" generally refers to a material suspected of containing the analyte. The test sample may, for instance, include materials obtained directly from a source, as well as materials pretreated using techniques, such as, but not limited to, filtration, precipitation, dilution, distillation, mixing, concentration, inactivation of interfering components, the addition of reagents, and so forth. The test sample may be derived from a biological source, such as a physiological fluid, including, blood, interstitial fluid, saliva, ocular lens fluid, cerebral spinal fluid, sweat, urine, milk, ascites fluid, mucous, synovial fluid, peritoneal fluid, vaginal fluid, amniotic fluid or the like. Besides physiological fluids, other liquid samples may be used, such as water, food products, and so forth. In addition, a solid material suspected of containing the analyte may also be used as the test sample.

Detailed Description

Reference now will be made in detail to various embodiments of the invention, one or more examples of which are set forth below. Each example is provided by way of explanation of the invention, not limitation of the invention. In fact, it will be apparent to those skilled in the art that various modifications and variations may be made in the present invention without departing from the scope or spirit of the invention. For instance, features illustrated or described as part of one embodiment, may be used on another embodiment to yield a still further embodiment. Thus, it is intended that the present invention covers such modifications and variations as come within the scope of the appended claims and their equivalents.

In general, the present invention is directed to a flow-through assay device for detecting the presence or quantity of an analyte residing in a test sample. The device utilizes a detection zone and compensation zone within which are immobilized capture reagents. The present inventor has discovered that the presence of a compensation zone may enable the detection of an analyte over extended concentration ranges. In particular, the signal from the compensation zone may compensate for the lost signal resulting from those probes that are embedded too deep within the interior of the assay device and/or those probes that exhibit self-quenching.

Referring to Fig. 1, for instance, one embodiment of a sandwich-type, flow-through assay device 20 that may be formed according to the present invention will now be described in more detail. As shown, the device 20 contains a porous membrane 23 optionally supported by a rigid material 21. In general, the porous membrane 23 may be made from any of a variety of materials through which the test sample is capable of passing. For example, the materials used to form the porous membrane 23 may include, but are not limited to, natural, synthetic, or naturally occurring materials that are synthetically modified, such as polysaccharides (e.g., cellulose materials such as paper and cellulose derivatives, such as cellulose acetate and nitrocellulose); polyether sulfone; polyethylene; nylon; polyvinylidene fluoride (PVDF); polyester; polypropylene; silica; inorganic materials, such as deactivated alumina, diatomaceous earth, $MgSO_4$, or other inorganic finely divided material uniformly dispersed in a porous polymer matrix,

with polymers such as vinyl chloride, vinyl chloride-propylene copolymer, and vinyl chloride-vinyl acetate copolymer; cloth, both naturally occurring (e.g., cotton) and synthetic (e.g., nylon or rayon); porous gels, such as silica gel, agarose, dextran, and gelatin; polymeric films, such as polyacrylamide; and the like. In one particular embodiment, the porous membrane 23 is formed from nitrocellulose and/or polyether sulfone materials. It should be understood that the term "nitrocellulose" refers to nitric acid esters of cellulose, which may be nitrocellulose alone, or a mixed ester of nitric acid and other acids, such as aliphatic carboxylic acids having from 1 to 7 carbon atoms.

The device 20 may also contain a wicking pad 28. The wicking pad 28 generally receives fluid that has migrated through the entire porous membrane 23. As is well known in the art, the wicking pad 28 may assist in promoting capillary action and fluid flow through the membrane 23.

To initiate the detection of an analyte within the test sample, a user may directly apply the test sample to a portion of the porous membrane 23 through which it may then travel in the direction illustrated by arrow “L” in Fig. 1. Alternatively, the test sample may first be applied to a sample pad (not shown) that is in fluid communication with the porous membrane 23. Some suitable materials that may be used to form the sample pad include, but are not limited to, nitrocellulose, cellulose, porous polyethylene pads, and glass fiber filter paper. If desired, the sample pad may also contain one or more assay pretreatment reagents, either diffusively or non-diffusively attached thereto.

In the illustrated embodiment, the test sample travels from the sample pad (not shown) to a conjugate pad 22 that is placed in communication with one end of the sample pad. The conjugate pad 22 is formed from a material through which the test sample is capable of passing. For example, in one embodiment, the conjugate pad 22 is formed from glass fibers. Although only one conjugate pad 22 is shown, it should be understood that other conjugate pads may also be used in the present invention.

To facilitate accurate detection of the presence or absence of an analyte within the test sample, a predetermined amount of detection probes are applied at various locations of the device 20. Any substance generally capable of generating a signal that is detectable visually or by an instrumental device may be used as

detection probes. Various suitable substances may include chromogens; catalysts; luminescent compounds (e.g., fluorescent, phosphorescent, etc.); radioactive compounds; visual labels, including colloidal metallic (e.g., gold) and non-metallic particles, dye particles, enzymes or substrates, or organic polymer latex particles; liposomes or other vesicles containing signal producing substances; and so forth. For instance, some enzymes suitable for use as detection probes are disclosed in U.S. Patent No. 4,275,149 to Litman, et al., which is incorporated herein in its entirety by reference thereto for all purposes. One example of an enzyme/substrate system is the enzyme alkaline phosphatase and the substrate nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate, or derivative or analog thereof, or the substrate 4-methylumbelliferyl-phosphate. Other suitable detection probes may be described in U.S. Patent Nos. 5,670,381 to Jou, et al. and 5,252,459 to Tarcha, et al., which are incorporated herein in their entirety by reference thereto for all purposes.

In some embodiments, the detection probes may contain a fluorescent compound that produces a detectable signal. The fluorescent compound may be a fluorescent molecule, polymer, dendrimer, particle, and so forth. Some examples of suitable fluorescent molecules, for instance, include, but are not limited to, fluorescein, europium chelates, phycobiliprotein, rhodamine and their derivatives and analogs.

The detection probes, such as described above, may be used alone or in conjunction with a microparticle (sometimes referred to as "beads" or "microbeads"). For instance, naturally occurring microparticles, such as nuclei, mycoplasma, plasmids, plastids, mammalian cells (e.g., erythrocyte ghosts), unicellular microorganisms (e.g., bacteria), polysaccharides (e.g., agarose), and so forth, may be used. Further, synthetic microparticles may also be utilized. For example, in one embodiment, latex microparticles that are labeled with a fluorescent or colored dye are utilized. Although any latex microparticle may be used in the present invention, the latex microparticles are typically formed from polystyrene, butadiene styrenes, styreneacrylic-vinyl terpolymer, polymethylmethacrylate, polyethylmethacrylate, styrene-maleic anhydride copolymer, polyvinyl acetate, polyvinylpyridine, polydivinylbenzene, polybutyleneterephthalate, acrylonitrile, vinylchloride-acrylates, and so forth, or an

aldehyde, carboxyl, amino, hydroxyl, or hydrazide derivative thereof. Other suitable microparticles may be described in U.S. Patent Nos. 5,670,381 to Jou, et al. and 5,252,459 to Tarcha, et al., which are incorporated herein in their entirety by reference thereto for all purposes. Commercially available examples of suitable fluorescent particles include fluorescent carboxylated microspheres sold by Molecular Probes, Inc. under the trade names "FluoSphere" (Red 580/605) and "TransfluoSphere" (543/620), as well as "Texas Red" and 5- and 6-carboxytetramethylrhodamine, which are also sold by Molecular Probes, Inc. In addition, commercially available examples of suitable colored, latex microparticles include carboxylated latex beads sold by Bang's Laboratory, Inc.

When utilized, the shape of the particles may generally vary. In one particular embodiment, for instance, the particles are spherical in shape. However, it should be understood that other shapes are also contemplated by the present invention, such as plates, rods, discs, bars, tubes, irregular shapes, etc. In addition, the size of the particles may also vary. For instance, the average size (e.g., diameter) of the particles may range from about 0.1 nanometers to about 1,000 microns, in some embodiments, from about 0.1 nanometers to about 100 microns, and in some embodiments, from about 1 nanometer to about 10 microns. For instance, "micron-scale" particles are often desired. When utilized, such "micron-scale" particles may have an average size of from about 1 micron to about 1,000 microns, in some embodiments from about 1 micron to about 100 microns, and in some embodiments, from about 1 micron to about 10 microns. Likewise, "nano-scale" particles may also be utilized. Such "nano-scale" particles may have an average size of from about 0.1 to about 10 nanometers, in some embodiments from about 0.1 to about 5 nanometers, and in some embodiments, from about 1 to about 5 nanometers.

In some instances, it is desired to modify the detection probes in some manner so that they are more readily able to bind to the analyte. In such instances, the detection probes may be modified with certain specific binding members that are adhered thereto to form conjugated probes. Specific binding members generally refer to a member of a specific binding pair, i.e., two different molecules where one of the molecules chemically and/or physically binds to the second molecule. For instance, immunoreactive specific binding members may

include antigens, haptens, aptamers, antibodies (primary or secondary), and complexes thereof, including those formed by recombinant DNA methods or peptide synthesis. An antibody may be a monoclonal or polyclonal antibody, a recombinant protein or a mixture(s) or fragment(s) thereof, as well as a mixture of an antibody and other specific binding members. The details of the preparation of such antibodies and their suitability for use as specific binding members are well known to those skilled in the art. Other common specific binding pairs include but are not limited to, biotin and avidin (or derivatives thereof), biotin and streptavidin, carbohydrates and lectins, complementary nucleotide sequences (including probe and capture nucleic acid sequences used in DNA hybridization assays to detect a target nucleic acid sequence), complementary peptide sequences including those formed by recombinant methods, effector and receptor molecules, hormone and hormone binding protein, enzyme cofactors and enzymes, enzyme inhibitors and enzymes, and so forth. Furthermore, specific binding pairs may include members that are analogs of the original specific binding member. For example, a derivative or fragment of the analyte, i.e., an analyte-analog, may be used so long as it has at least one epitope in common with the analyte.

The specific binding members may generally be attached to the detection probes using any of a variety of well-known techniques. For instance, covalent attachment of the specific binding members to the detection probes (e.g., particles) may be accomplished using carboxylic, amino, aldehyde, bromoacetyl, iodoacetyl, thiol, epoxy and other reactive or linking functional groups, as well as residual free radicals and radical cations, through which a protein coupling reaction may be accomplished. A surface functional group may also be incorporated as a functionalized co-monomer because the surface of the detection probe may contain a relatively high surface concentration of polar groups. In addition, although detection probes are often functionalized after synthesis, in certain cases, such as poly(thiophenol), the microparticles are capable of direct covalent linking with a protein without the need for further modification. For example, referring to Fig. 2, one embodiment of the present invention for covalently conjugating a particle-containing detection probe is illustrated. As shown, the first step of conjugation is activation of carboxylic groups on the probe surface using carbodiimide. In the second step, the activated carboxylic acid groups are reacted

with an amino group of an antibody to form an amide bond. The activation and/or antibody coupling may occur in a buffer, such as phosphate-buffered saline (PBS) (e.g., pH of 7.2) or 2-(N-morpholino) ethane sulfonic acid (MES) (e.g., pH of 5.3). As shown, the resulting detection probes may then be blocked with ethanolamine, for instance, to block any remaining activated sites. Overall, this process forms a conjugated detection probe, where the antibody is covalently attached to the probe. Besides covalent bonding, other attachment techniques, such as physical adsorption, may also be utilized in the present invention.

Referring again to Fig. 1, the assay device 20 may also contain a detection zone 31 within which is immobilized a first capture reagent that is capable of binding to the conjugated detection probes. For example, in some embodiments, the first capture reagent may be a biological capture reagent. Such biological capture reagents are well known in the art and may include, but are not limited to, antigens, haptens, protein A or G, neutravidin, avidin, streptavidin, captavidin, primary or secondary antibodies (e.g., polyclonal, monoclonal, etc.), and complexes thereof. In many cases, it is desired that these biological capture reagents are capable of binding to a specific binding member (e.g., antibody) present on the detection probes. The first capture reagent serves as a stationary binding site for complexes formed between the analyte and conjugated detection probes. Specifically, analytes, such as antibodies, antigens, etc., typically have two or more binding sites (e.g., epitopes). Upon reaching the detection zone 31, one of these binding sites is occupied by the specific binding member of the conjugated probe. However, the free binding site of the analyte may bind to the immobilized capture reagent. Upon being bound to the immobilized capture reagent, the complexed probes form a new ternary sandwich complex.

The detection zone 31 may generally provide any number of distinct detection regions so that a user may better determine the concentration of a particular analyte within a test sample. Each region may contain the same capture reagents, or may contain different capture reagents for capturing multiple analytes. For example, the detection zone 31 may include two or more distinct detection regions (e.g., lines, dots, etc.). The detection regions may be disposed in the form of lines in a direction that is substantially perpendicular to the flow of the test sample through the assay device 20. Likewise, in some embodiments, the

detection regions may be disposed in the form of lines in a direction that is substantially parallel to the flow of the test sample through the assay device.

Referring again to Fig. 1, the porous membrane 23 also contains a compensation zone 35 positioned downstream from the detection zone 31. The compensation zone 35 generally provides a single distinct region (e.g., line, dot, etc.), although multiple regions are certainly contemplated by the present invention. For instance, in the illustrated embodiment, a single line is utilized. The compensation zone 35 may be disposed in a direction that is substantially perpendicular to the flow of the test sample through the device 20. Likewise, in some embodiments, the zone 35 may be disposed in a direction that is substantially parallel to the flow of the test sample through the device 20.

Regardless of its configuration, a second capture reagent is immobilized on the membrane 35 within the compensation zone 35. The second capture reagent serves as a stationary binding site for any conjugated detection probes and/or analyte/conjugated probe complexes that do not bind to the first capture reagent at the detection zone 31. Because it is desired that the second capture reagent bind to both complexed and uncomplexed conjugated detection probes, the second capture reagent is normally different than the first capture reagent. In one embodiment, the second capture reagent is a biological capture reagent (e.g., antigens, haptens, protein A or G, neutravidin, avidin, streptavidin, primary or secondary antibodies (e.g., polyclonal, monoclonal, etc.), and complexes thereof) that is different than the first capture reagent. For example, the first capture reagent may be a monoclonal antibody (e.g., CRP Mab1), while the second capture reagent may be avidin (a highly cationic 66,000-dalton glycoprotein), streptavidin (a nonglycosylated 52,800-dalton protein), neutravidin (a deglysolated avidin derivative), and/or captavidin (a nitrated avidin derivative). In this embodiment, the second capture reagent may bind to biotin, which is biotinylated or contained on detection probes conjugated with a monoclonal antibody different than the monoclonal antibody of the first capture reagent (e.g., CRP Mab2).

In addition, it may also be desired to utilize various non-biological materials for the second capture reagent of the compensation zone 35. In many instances, such non-biological capture reagents may be particularly desired to better ensure that all of the remaining conjugated detection probes and/or analyte/conjugated

probe complexes are immobilized at the compensation zone 35. For instance, in some embodiments, the second capture reagent may include a polyelectrolyte.

The polyelectrolytes may have a net positive or negative charge, as well as a net charge that is generally neutral. For instance, some suitable examples of polyelectrolytes having a net positive charge include, but are not limited to, polylysine (commercially available from Sigma-Aldrich Chemical Co., Inc. of St. Louis, Missouri), polyethyleneimine; epichlorohydrin-functionalized polyamines and/or polyamidoamines, such as poly(dimethylamine-co-epichlorohydrin); polydiallyldimethyl-ammonium chloride; cationic cellulose derivatives, such as cellulose copolymers or cellulose derivatives grafted with a quaternary ammonium water-soluble monomer; and so forth. In one particular embodiment, CelQuat® SC-230M or H-100 (available from National Starch & Chemical, Inc.), which are cellulosic derivatives containing a quaternary ammonium water-soluble monomer, may be utilized. Moreover, some suitable examples of polyelectrolytes having a net negative charge include, but are not limited to, polyacrylic acids, such as poly(ethylene-co-methacrylic acid, sodium salt), and so forth. It should also be understood that other polyelectrolytes may also be utilized, such as amphiphilic polyelectrolytes (i.e., having polar and non-polar portions). For instance, some examples of suitable amphiphilic polyelectrolytes include, but are not limited to, poly(styryl-b-N-methyl 2-vinyl pyridnium iodide) and poly(styryl-b-acrylic acid), both of which are available from Polymer Source, Inc. of Dorval, Canada.

Although any polyelectrolyte may generally be used, the polyelectrolyte selected for a particular application may vary depending on the nature of the detection probes, the porous membrane, and so forth. In particular, the distributed charge of a polyelectrolyte allows it to bind to substances having an opposite charge. Thus, for example, polyelectrolytes having a net positive charge are often better equipped to bind with detection probes that are negatively charged, while polyelectrolytes that have a net negative charge are often better equipped to bind to detection probes that are positively charged. Thus, in such instances, the ionic interaction between these molecules allows the required binding to occur within the compensation zone 35. Nevertheless, although ionic interaction is primarily utilized to achieve the desired binding in the compensation zone 35, it has also been discovered that polyelectrolytes may bind with detection probes having a

similar charge.

Because the polyelectrolyte is designed to bind to detection probes, it is typically desired that the polyelectrolyte be substantially non-diffusively immobilized on the surface of the porous membrane 23. Otherwise, the detection probes would not be readily detectable by a user. Thus, the polyelectrolytes may be applied to the porous membrane 23 in such a manner that they do not substantially diffuse into the matrix of the porous membrane 23. In particular, the polyelectrolytes typically form an ionic and/or covalent bond with functional groups present on the surface of the porous membrane 23 so that they remain immobilized thereon. Although not required, the formation of covalent bonds between the polyelectrolyte and the porous membrane 23 may be desired to more permanently immobilize the polyelectrolyte thereon.

For example, in one embodiment, the monomers used to form the polyelectrolyte are first formed into a solution and then applied directly to the porous membrane 23. Various solvents (e.g., organic solvents, water, etc.) may be utilized to form the solution. Once applied, the polymerization of the monomers is initiated using heat, electron beam radiation, free radical polymerization, and so forth. In some instances, as the monomers polymerize, they form covalent bonds with certain functional groups of the porous membrane 23, thereby immobilizing the resulting polyelectrolyte thereon. For example, in one embodiment, an ethyleneimine monomer may form a covalent bond with a carboxyl group present on the surface of some porous membranes (e.g., nitrocellulose).

In another embodiment, the polyelectrolyte may be formed prior to application to the porous membrane 23. If desired, the polyelectrolyte may first be formed into a solution using organic solvents, water, and so forth. Thereafter, the polyelectrolytic solution is applied directly to the porous membrane 23 and then dried. Upon drying, the polyelectrolyte may form an ionic bond with certain functional groups present on the surface of the porous membrane 23 that have a charge opposite to the polyelectrolyte. For example, in one embodiment, positively-charged polyethyleneimine may form an ionic bond with negatively-charged carboxyl groups present on the surface of some porous membranes (e.g., nitrocellulose).

In addition, the polyelectrolyte may also be crosslinked to the porous

membrane 23 using various well-known techniques. For example, in some embodiments, epichlorohydrin-functionalized polyamines and/or polyamidoamines may be used as a crosslinkable, positively-charged polyelectrolyte. Examples of these materials are described in U.S. Pat. Nos. 3,700,623 to Keim and 3,772,076 to Keim, 4,537,657 to Keim, which are incorporated herein in their entirety by reference thereto for all purposes and are believed to be sold by Hercules, Inc., Wilmington, Del. under the Kymene™ trade designation. For instance, Kymene™ 450 and 2064 are epichlorohydrin-functionalized polyamine and/or polyamidoamine compounds that contain epoxide rings and quaternary ammonium groups that may form covalent bonds with carboxyl groups present on certain types of porous membranes (e.g., nitrocellulose) and crosslink with the polymer backbone of the porous membrane when cured. In some embodiments, the crosslinking temperature may range from about 50°C to about 120°C and the crosslinking time may range from about 10 to about 600 seconds.

Although various techniques for non-diffusively immobilizing polyelectrolytes on the porous membrane 23 have been described above, it should be understood that any other technique for non-diffusively immobilizing polyelectrolytic compounds may be used in the present invention. In fact, the aforementioned methods are only intended to be exemplary of the techniques that may be used in the present invention. For example, in some embodiments, certain components may be added to the polyelectrolyte solution that may substantially inhibit the diffusion of such polyelectrolytes into the matrix of the porous membrane 23.

Regardless of the material from which the second capture reagent is formed, the compensation zone 35 may improve the analyte detection range of the assay device 20. This phenomenon is illustrated graphically in Fig. 3. As shown, the intensity of the signal at the detection zone 31 ("I_{det}") initially increases as more of the analyte is captured at the detection zone 31. Ideally, the measured detection signal intensity would continue to increase linearly for higher analyte concentrations. However, optical detection methods (e.g., fluorescence and reflectance) do always not provide such an ideal measurement, particularly at relatively high detection probe concentrations. Specifically, at some point, the detection zone 31 would be unable to signal the further accumulation of detection probes. As a result, the signal at the detection zone 31 would level off or even

decrease. For instance, as shown in Fig. 3, I_{det} may begin to level off at an analyte concentration of "A_{sat}" as the analyte concentration further increases.

In accordance with the present invention, however, the intensity signal at the compensation zone ("I_{com}") may be measured to account for the inability of the detection zone 31 to respond to higher analyte concentrations. When no analyte is present, I_{com} will be at its maximum intensity because all of the conjugated detection probes will bind to the compensation zone 35. As the analyte concentration is increased, I_{com} likewise decreases due to the retention of a greater number of analyte/conjugated probe complexes by the detection zone 31. As a result of the inversely proportional relationship between the detection and compensation zone signal intensities described above, the present inventor has discovered that the concentration of an analyte may be more effectively measured over an extended range by comparing the signal intensity at both the detection and compensation zones. Specifically, the total amount of detection probes is predetermined (e.g., empirically). Because a predetermined amount of detection probes are present, the amount of detection probes captured at the compensation zone 35 is inversely proportional to the amount of detection probes at the detection zone 31. Thus, the amount of detection probes captured at the compensation zone 35 may be measured relatively accurately, even when large amounts of detection probes are captured at the detection zone 31 and the amount of such amount of such detection probes cannot be measured accurately. For example, in one embodiment, the amount of analyte is directly proportional to the ratio of I_{det} to I_{com}. Based upon the intensity range in which the detection and compensation zones fall, the general concentration range for the analyte may be determined. If desired, the ratio of I_{det} to I_{com} may be plotted versus the analyte concentration for a range of known analyte concentrations to generate an intensity curve. To determine the quantity of analyte in an unknown test sample, the signal ratio may then be converted to analyte concentration according to the intensity curve. It should be noted that the capturing efficiency of the complexed and uncomplexed conjugated detection probes is generally the same for any given sample. Accordingly, the variation in capturing efficiency is not believed to significantly interfere with the results from sample-to-sample because the ratio of intensities (i.e., I_{det}/I_{com}) is used instead of absolute signal intensity. It should also be noted

that alternative mathematical relationships between I_{det} and I_{com} may be plotted versus the analyte concentration to generate the calibration curve. For example, in one embodiment, the value of $I_{det} / (I_{det} + I_{com})$ may be plotted versus analyte concentration to generate the intensity curve.

Although the detection zone 31 and compensation zone 35 may indicate the presence of an analyte, it is often difficult to accurately determine the relative concentration of the analyte within the test sample under actual test conditions. Thus, the assay device 20 may also include a calibration zone 32. In this embodiment, the calibration zone 32 is formed on the porous membrane 23 and is positioned downstream from the detection zone 31 and compensation zone 35. Alternatively, however, the calibration zone 32 may also be positioned upstream from the detection zone 31 and/or compensation zone 35.

The calibration zone 32 is provided with a third capture reagent that is capable of binding to calibration probes that pass through the length of the membrane 23. The calibration probes may be formed from the same or different materials as the detection probes, and may be conjugated with a specific binding member as described above. Generally speaking, the calibration probes are selected in such a manner that they do not bind to the first or second capture reagent at the detection zone 31 and compensation zone 35. The third capture reagent may also be the same or different than the capture reagents used in the detection zone 31 or compensation zone 35. For example, in one embodiment, the third capture reagent is a biological capture reagent, such as antigens, haptens, protein A or G, neutravidin, avidin, streptavidin, captavidin, primary or secondary antibodies, or complexes thereof. Moreover, similar to the detection zone 31 and compensation zone 35, the calibration zone 32 may also provide any number of distinct calibration regions in any direction so that a user may better determine the concentration of a particular analyte within a test sample.

The calibration zone 32 may improve the accuracy of the detected analyte. The calibration zone 32 may also eliminate requirement of separate calibration for measurements carried out at a different time under different conditions. The total amount of the calibration probes and the total amount of the third capture reagent on the calibration zone 32 is predetermined. Thus, the amount of the captured calibration probes and the resulting calibration signal ideally fluctuates in a manner

similar to what would occur at the detection zone 31 based on changing assay conditions, e.g., temperature fluctuation. Desirably, the third capture reagent has a similar degradation profile to that of the first capture reagent at the detection zone 31. The calibration probes may also have a similar degradation profile to that of the detection probes. The signal fluctuation of both the detection probes and the calibration probes is ideally the same or similar with changed conditions.

Thus, the calibration zone 32 may be used to calibrate the intensities of the detection zone 31 and compensation zone 35 under different assay conditions. For example, referring again to Fig. 3, the amount of analyte may be directly proportional to the ratio of I_{det} to the product of the calibration intensity ("I_{cal}") and I_{com} (i.e., $I_{det}/(I_{cal})(I_{com})$). If desired, this may be plotted versus the analyte concentration for a range of known analyte concentrations to generate a calibration curve. To determine the quantity of analyte in an unknown test sample, the signal ratio may then be converted to analyte concentration according to the calibration curve. It should also be noted that alternative mathematical relationships may be plotted versus the analyte concentration to generate the calibration curve.

Referring to Fig. 4, one embodiment of a method for detecting the presence of an analyte utilizing fluorescent probes will now be described in more detail. Initially, a test sample containing an analyte A is applied to the sample pad. From the sample pad, the test sample travels in the direction "L" to the conjugate pad 22, where the analyte A mixes with conjugated fluorescent detection probes 41 and fluorescent calibration probes 43 (may or may not be conjugated). Although the use of fluorescence is utilized in this particular embodiment, it should be understood that other optical detection techniques, such as phosphorescence, reflectance, etc., are equally suitable for use in the present invention. For example, in one embodiment, as is well known in the art, a reflectance spectrophotometer or reader may be utilized to detect the presence of probes that exhibit a visual color (e.g. dyed latex microparticles). One suitable reflectance reader is described, for instance, in U.S. Patent App. Pub. No. 2003/0119202 to Kaylor, et al., which is incorporated herein in its entirety by reference thereto for all purposes.

Nevertheless, in the embodiment illustrated in Fig. 4, the analyte A binds with the conjugated fluorescent detection probes 41 to form analyte/conjugated

probe complexes 49. At the detection zone 31, these complexes 49 are captured by a first capture reagent 90. Any uncomplexed conjugated fluorescent detection probes 41 and/or unbound analyte/conjugated probe complexes 49 then travel to the compensation zone 35 where they bind to a second capture reagent (not shown). Finally, the fluorescent calibration probes 43 travel through both the detection zone 31 and compensation zone 35 to bind with a third capture reagent (not shown) at the calibration zone 32.

Once captured, the fluorescence signal of the probes at the detection zone 31, compensation zone 35, and calibration zone 32 may be measured using fluorescence detection. Fluorescence is the result of a three-stage process that occurs in certain fluorescent compounds. In the first stage, energy is supplied by an external source, such as an incandescent lamp or a laser and absorbed by the fluorescent compound, creating an excited electronic singlet state. In the second stage, the excited state exists for a finite time during which the fluorescent compound undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. During this time, the energy of the excited state is partially dissipated, yielding a relaxed state from which fluorescence emission originates. The third stage is the fluorescence emission stage wherein energy is emitted, returning the fluorescent compound to its ground state. The emitted energy is lower than its excitation energy (light or laser) and thus of a longer wavelength. This shift or difference in energy or wavelength allows the emission energy to be detected and isolated from the excitation energy.

Fluorescence detection generally utilizes wavelength filtering to isolate the emission photons from the excitation photons, and a detector that registers emission photons and produces a recordable output, usually as an electrical signal or a photographic image. There are generally four recognized types of detectors: spectrofluorometers and microplate readers; fluorescence microscopes; fluorescence scanners; and flow cytometers. One suitable fluorescence detector for use with the present invention is a FluoroLog III Spectrofluorometer, which is sold by SPEX Industries, Inc. of Edison, New Jersey.

If desired, a technique known as "time-resolved fluorescence detection" may also be utilized in the present invention. Time-resolved fluorescence detection is designed to reduce background signals from the emission source or

from scattering processes (resulting from scattering of the excitation radiation) by taking advantage of the fluorescence characteristics of certain fluorescent materials, such as lanthanide chelates of europium (Eu (III)) and terbium (Tb (III)). Such chelates may exhibit strongly red-shifted, narrow-band, long-lived emission after excitation of the chelate at substantially shorter wavelengths. Typically, the chelate possesses a strong ultraviolet absorption band due to a chromophore located close to the lanthanide in the molecule. Subsequent to light absorption by the chromophore, the excitation energy may be transferred from the excited chromophore to the lanthanide. This is followed by a fluorescence emission characteristic of the lanthanide. The use of pulsed excitation and time-gated detection, combined with narrow-band emission filters, allows for specific detection of the fluorescence from the lanthanide chelate only, rejecting emission from other species present in the sample that are typically shorter-lived or have shorter wavelength emission. Other time-resolved techniques for measuring fluorescence are described in U.S. Patent No. 5,585,279 to Davidson and 5,637,509 to Hemmila, et al., which are incorporated herein in their entirety by reference thereto for all purposes.

Regardless of the technique used to measure fluorescence, the absolute amount of the analyte may be ascertained by comparing the fluorescence signal at the detection zone 31 with the fluorescence signal at the compensation zone 35, and optionally with the fluorescent signal at the calibration zone 32. For example, as indicated above, the amount of analyte may be determined by the ratio of $I_{det}/(I_{cal})(I_{com})$, and converting this ratio to an analyte concentration using a previously ascertained calibration curve.

Although various embodiments of device configurations have been described above, it should be understood, that a device of the present invention may generally have any configuration desired, and need not contain all of the components described above. Various other device configurations, for instance, are described in U.S. Patent Nos. 5,395,754 to Lambotte, et al.; 5,670,381 to Jou, et al.; and 6,194,220 to Malick, et al., which are incorporated herein in their entirety by reference thereto for all purposes. Various assay formats may also be used to test for the presence or absence of an analyte using the assay device of the present invention. For instance, in the embodiment described above, a "sandwich"

format is utilized. Other examples of such sandwich-type assays are described by U.S. Patent Nos. 4,168,146 to Grubb, et al. and 4,366,241 to Tom, et al., which are incorporated herein in their entirety by reference thereto for all purposes. In addition, other formats, such as "competitive" formats, may also be utilized. Examples of competitive immunoassay devices are described in U.S. Patent Nos. 4,235,601 to Deutsch, et al., 4,442,204 to Liotta, and 5,208,535 to Buechler, et al., which are incorporated herein in their entirety by reference thereto for all purposes.

The present inventor has discovered that the presence of a compensation zone on an assay device may enable the detection of an analyte over extended concentration ranges in a simple, efficient, and cost-effective manner. In particular, the compensation zone may compensate for the lost signals that would otherwise result from the limitations of optical detection techniques.

The present invention may be better understood with reference to the following examples.

EXAMPLE 1

Conjugated fluorescent detection probes were formed in the following manner. Carboxylated latex particles were encapsulated with europium chelates having a particle size of 0.20 micrometers, a 0.5% solids concentration, and exhibiting fluorescence at an emission wavelength of 615 nanometers when excited at a wavelength of 370 nanometers. The particles were obtained from Molecular Probes, Inc. and designated as "Eu-P."

Initially, 500 microliters of the particles were washed once with 1 milliliter of a carbonate buffer and twice with 2-[N-morpholino]ethanesulfonic acid (MES) buffer (pH: 6.1, 20 millimolar) using a centrifuge. The washed particles were re-suspended in 250 microliters of MES. Thereafter, 3 milligrams of carbodiimide (Polysciences, Inc.) was dissolved in 250 microliters of MES and added to the suspended particles. The mixture was allowed to react at room temperature for 30 minutes on a shaker. The activated particles were then washed twice with a borate buffer (Polysciences, Inc) and re-suspended in 250 microliters of borate buffer. 30 microliters of C-protein monoclonal antibody (CRP Mab1) (3.4 milligrams per milliliter, A#5811 from BiosPacific, Inc.) was then added to the particle suspensions. The mixture was allowed to react at room temperature overnight on an end-over-end shaker. During the period of reaction, the

suspensions were bath-sonicated twice. The particles were then collected and incubated in 250 microliters of 0.1 molar ethanolamine (Polysciences Inc.) under gentle shaking for 15 minutes. The particles were washed twice with hepes buffer (N-[2-hydroxyethyl]piperazine-N'-(2-ethanesulfonic acid) (20 millimolar, pH: 7.2). The washed conjugates were suspended in 1 milliliter of Hepes buffer and stored at 4°C.

EXAMPLE 2

Conjugated fluorescent calibration probes were formed as described in Example 1, except that CRP Mab1 was replaced with Rabbit anti Goat IgG (Cat# 41-RG15 from BiosPacific, Inc. Inc.) or Goat anti Rabbit IgG (Cat# 41-GR30 from BiosPacific, Inc. Inc). The conjugated fluorescent calibration probes were designated as "Eu-P 41-RG15" and "Eu-P 41-GR30", respectively.

EXAMPLE 3

The ability to form a lateral flow assay device with a detection zone and a compensation zone was demonstrated. A nitrocellulose porous membrane (HF 12002 from Millipore, Inc.) having a length of approximately 30 centimeters was laminated onto supporting cards. Goldline™ (a polylysine solution obtained from British Biocell International) was stripped onto the membrane to form a compensation zone. In addition, monoclonal antibody for C-reactive protein (CRP Mab2) (A#5804, available from BiosPacific, Inc., concentration of 1 milligram per milliliter) was immobilized on the porous membrane to form a detection zone. The membrane samples were then dried for 1 hour at a temperature of 37°C.

A conjugate pad was prepared as described below. 250 microliters of Eu-P CRP conjugated fluorescent detection particles of Example 1 (concentration of 2.5 milligrams per milliliter in Hepes buffer) was mixed with 375 microliters of Tween 20 (2%, available from Aldrich) and 375 microliters of sucrose in water (10%). The mixture was bath-sonicated for 20 minutes. The suspension was then loaded onto a 15-centimeter long glass fiber conjugate pad (Millipore Co.). The glass fiber pad was then dried at 37°C for 2 hours.

A sample pad was prepared by loading 900 microliters of Tween 20 (0.5%) onto a 15-centimeter long glass fiber sample pad (Millipore Co.), and then drying the pad at 37°C for 2 hours. A cellulose wicking pad (Millipore Co.), the sample pad, and conjugate pad were then laminated onto the porous membrane. The

laminated full card was then cut into 4-millimeter wide lateral flow assay devices.

EXAMPLE 4

The ability to detect the presence of an analyte using a lateral flow assay device was demonstrated. Specifically, eleven (11) of the assay devices prepared as described in Example 3 were tested. 55 microliters of diluted human blood (diluted by 100 times) was spiked with eleven (11) different CRP concentrations, ranging from 0, 0.2, 0.5, 1, 2, 10, 40, 100, 200, 500, and 2000 nanograms per milliliter, and applied to separate sample pads. The devices were allowed to develop for 30 minutes.

The fluorescence for the detection zone and calibration zone was measured. Specifically, upon completion of the assay, each lateral flow device was mounted onto a sample holder of a Fluorolog III Spectrofluorometer (available from SA Instruments, Inc.) using tape. The detection and compensation zones each fit into a rectangular hole in the holder so that the excitation beam would shine directly on the zone while the rest of the device was remained blocked from the excitation beam. Time-resolved fluorescence techniques were used. Specifically, the following experiment parameters were used: (1) the angle of the excitation beam to the surface normal of the devices was 70°C; the detection mode was front face; the slit width was 5 nanometer; (4) the number of scan was 1; (5) the excitation wavelength was 370 nanometers; (6) the emission wavelength was collected at 615 nanometers; (7) the sample window was 3 milliseconds (ms); (8) the initial delay was 0.04 ms; (9) the time-per-flash was 50 ms; and (10) the number of flashes was 10.

The intensity at the detection zone for CRP concentrations of 0, 0.2, 0.5, 1, 2, 10, 40, 100, 200, 500, and 2000 nanograms per milliliter was determined to be 10.4K, 12.4K, 14.7K, 15.8K, 27.0K, 61.7K, 99.1K, 145.8K, 190.4K, 214.5K, 206.0K, respectively. The intensity at the compensation zone for CRP concentrations of 0, 0.2, 0.5, 1, 2, 10, 40, 100, 200, 500, and 2000 nanograms per milliliter was determined to be 280.9K, 216.3K, 165.0K, 187.5K, 170.0K, 123.7K, 65.4K, 56.0K, 8.2K, 3.9K, 2.3K, respectively. The intensity of the detection zone initially increased, but leveled off at a CRP concentration of about 200 to 500 nanograms per milliliter. The intensity of the compensation zone continued to decrease, even at a CRP concentration as high as 2000 nanograms per milliliter.

Therefore, the ratio of the intensity at the detection zone to the intensity at the compensation zone would more accurately present the true CRP concentration for CRP concentrations higher than about 200 nanograms per milliliter.

EXAMPLE 5

The ability to form a lateral flow assay device with a detection zone, a calibration zone, and a compensation zone was demonstrated. A nitrocellulose porous membrane (HF 12002 from Millipore, Inc.) having a length of approximately 30 centimeters was laminated onto supporting cards. Goldline™ (a polylysine solution obtained from British Biocell International) was stripped onto the membrane to form a compensation zone. Monoclonal antibody for C-reactive protein (CRP Mab2) (A#5804, available from BiosPacific, Inc., concentration of 1 milligram per milliliter) was immobilized on the porous membrane to form a detection zone. In addition, Rabbit anti prolactin antibody (A#5804, available from BiosPacific, Inc., concentration of 1.8 milligram per milliliter) was immobilized between the detection zone and the compensation zone on the porous membrane to form a calibration zone. The membrane samples were then dried for 1 hour at a temperature of 37°C.

250 microliters of the Eu-P CRP particles of Example 1 (concentration of 2.5 milligrams per milliliter in Hepes buffer) and 100 microliters of the Eu-P GR30 (2.5 milligrams per milliliter in Hepes buffer) particles of Example 2 were mixed with 300 microliters of Tween 20 (2%, available from Aldrich) and 300 microliters of sucrose in water (10%). The Eu-P CRP particles were used as detection probes, while the EU-P GR30 particles were used as calibration probes. The mixture was bath-sonicated for 20 minutes. The suspension was then loaded onto a 15-centimeter long glass fiber conjugate pad (Millipore Co.). The glass fiber pad was then dried at 37°C for 2 hours.

A sample pad was prepared by loading 900 microliters of Tween 20 (0.5%) onto a 15-centimeter long glass fiber sample pad (Millipore Co.), and then drying the pad at 37°C for 2 hours. A cellulose wicking pad (Millipore Co.), the sample pad, and conjugate pad were then laminated onto the porous membrane. The laminated full card was then cut into 4-millimeter wide lateral flow assay devices.

EXAMPLE 6

The ability to detect the presence of an analyte using a lateral flow assay

device was demonstrated. Specifically, nine (9) of the assay devices prepared as described in Example 5 were tested. 50 microliters of Hepes buffer was spiked with nine (9) different CRP concentrations, ranging from 0, 5, 20, 100, 500, 1000, 2000, 5000, and 10000 nanograms per milliliter, and applied to separate sample pads. The devices were allowed to develop for 30 minutes.

The time-gated fluorescence intensity was measured as described in Example 4, with the exception that the delay time was 0.04 milliseconds. The intensity at the detection zone for CRP concentrations of 0, 5, 20, 100, 500, 1000, 2000, 5000 and 10000 nanograms per milliliter was determined to be 26.7K, 39.0K, 47.0K, 109K, 159K, 186K, 217K, 219K, 193K, respectively. The intensity at the calibration zone for CRP concentrations of 0, 5, 20, 100, 500, 1000, 2000, 5000 and 10000 nanograms per milliliter was determined to be 96.6K, 136K, 101K, 119K, 103K, 88.7K, 86.8K, 88.1K, 87.9K, respectively. The intensity at the compensation zone for CRP concentrations of 0, 5, 20, 100, 500, 1000, 2000, 5000 and 10000 nanograms per milliliter was determined to be 123K, 146K, 93.6K, 158K, 131K, 81.8K, 69.3K, 54.1K, 34.0K, respectively. As indicated, the intensity at the detection zone initially increased, but then leveled off at a CRP concentration of about 2000 nanograms per milliliter, while the intensity at the compensation zone remained initially remained constant before beginning to decrease at a CRP concentration of about 1000 nanograms per milliliter. The intensity at the calibration zone remained relatively constant. Therefore, the ratio of the intensity at the detection zone to the intensity at the compensation zone, calibrated by the intensity at the calibration zone, would more accurately present the true CRP concentration for CRP concentrations of 2000 nanograms per milliliter or higher.

EXAMPLE 7

The ability to form a half lateral flow assay device with a detection zone, a calibration zone, and a compensation zone was demonstrated. A nitrocellulose porous membrane (HF 12002 from Millipore, Inc.) having a length of approximately 30 centimeters was laminated onto supporting cards. Goldline™ (a polylysine solution obtained from British Biocell International) was stripped onto the membrane to form a compensation zone. Monoclonal antibody for C-reactive protein (CRP Mab2) (A#5804, available from BiosPacific, Inc., concentration of 1

milligram per milliliter with 1 milligram of trehalose per milliliter) was immobilized on the porous membrane to form a detection zone. In addition, Rabbit anti prolactin antibody (A#5804, available from BiosPacific, Inc., concentration of 1.8 milligrams per milliliter) was immobilized between the detection zone and the compensation zone on the porous membrane to form a calibration zone. The membrane samples were then dried for 1 hour at a temperature of 37°C.

80 microliters of gold particles conjugated with Goat anti-Rabbit IgG (10 nanometer particle size, from Sigma) ("calibration probes") and 50 microliters of gold particles conjugated with CRP Mab1 (40 nanometer particle size, from British Biocell International) ("detection probes) were mixed with 280 microliters of water and 200 microliters of sucrose in water (10%). The suspension was then loaded onto a 10-centimeter long glass fiber conjugate pad (Millipore Co.). The glass fiber pad was then dried at 37°C for 2 hours. A sample pad was prepared by loading 300 microliters of Tween 20 (0.5%) and 1200 microliters of water onto a 10-centimeter cellulose pad (Millipore Co.), then drying the pad at 37°C for 2 hours. A cellulose wicking pad (Millipore Co.), the sample pad, and conjugate pad were then laminated onto the porous membrane. The laminated full card was then cut into 4-millimeter wide lateral flow assay devices.

EXAMPLE 8

The ability to detect the presence of an analyte using a lateral flow assay device was demonstrated. Specifically, ten (10) of the assay devices prepared as described in Example 7 were tested. 60 microliters of Hepes buffer was spiked with ten (10) different CRP concentrations, ranging from 0, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 nanograms per milliliter, and applied to separate sample pads. The devices were allowed to develop for 30 minutes.

The reflectance intensity was measured using a reflectance reader. The reflectance intensity at the detection zone for CRP concentrations of 0, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 nanograms per milliliter was determined to be 0, 0, 0, 0.0498, 0.0806, 0.4433, 1.418, 2.347, 2.407, and 2.402, respectively. The reflectance intensity at the calibration zone for CRP concentrations of 0, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 nanograms per milliliter was determined to be 1.072, 0.9650, 0.9752, 1.010, 0.9993, 0.8954, 1.030, 1.020, 1.035, and 1.070, respectively. The intensity at the compensation zone for CRP concentrations of 0,

5, 10, 20, 50, 100, 200, 500, 1000, and 2000 nanograms per milliliter was determined to be 1.414, 1.167, 1.345, 1.312, 1.045, 1.241, 1.331, 0.843, 0.6169, and 0.4608, respectively. As indicated, the reflectance intensity at the detection zone initially increased, and then leveled off at about a CRP concentration of about 500 nanograms per milliliter, while the intensity at the compensation zone initially remained relatively constant before beginning to decrease at a CRP concentration of about 200 nanograms per milliliter. The intensity at the calibration zone remained relatively constant. Therefore, the ratio of the reflectance intensity at the detection zone to the intensity at the compensation zone, calibrated by the intensity at the calibration zone, would more accurately present the true CRP concentration for CRP concentrations of 500 nanograms per milliliter or higher.

While the invention has been described in detail with respect to the specific embodiments thereof, it will be appreciated that those skilled in the art, upon attaining an understanding of the foregoing, may readily conceive of alterations to, variations of, and equivalents to these embodiments. Accordingly, the scope of the present invention should be assessed as that of the appended claims and any equivalents thereto.